# THE ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE EXPRESSION IN THE DEVELOPMENT OF PULMONARY HYPERTENSION IN CHRONICALLY HYPOXIC INFANT SWINE

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***Objective:*** **Our goal was to determine the role of pulmonary endothelial nitric oxide synthase expression in the development of pulmonary hypertension in infants with congenital cyanotic heart disease.** ***Methods:*** **Two groups of 4-week-old piglets were studied. In one group, the piglets were raised in an environment of 10% oxygen from 2 days of age (cyanotic, $n = 6$), and in the other group the piglets were raised at room air (control, $n = 5$). Pulmonary hemodynamics were measured in vivo for each animal, and peripheral lung biopsy specimens were obtained for Western blot analysis with the use of antiendothelial nitric oxide synthase antibody and for activity analysis with the use of the tritiated L-arginine assay.** ***Results:*** **The piglets in the chronically hypoxic group had significant increases in mean pulmonary arterial pressure (44.0 ± 3.8 mm Hg vs 14.8 ± 1.2 mm Hg in controls, $p = 0.0007$) and pulmonary vascular resistance (7272.0 ± 871.1 dyne · cm · sec$^{-5}$ vs 1844.5 ± 271.2 dyne · cm · sec$^{-5}$ in controls, $p = 0.002$). These changes in the pulmonary hemodynamics of the hypoxic piglets were accompanied by a twofold increase in the expression of pulmonary endothelial nitric oxide synthase ($p = 0.0043$) but no corresponding increase in nitric oxide synthase activity.** ***Conclusions:*** **Raising infant piglets in an environment of 10% oxygen for 4 weeks results in significant pulmonary arterial hypertension accompanied by increased expression of nitric oxide synthase within the lung endothelium. Furthermore, the increased levels of nitric oxide synthase within the lungs of the hypoxic swine were not accompanied by a proportional increase in enzyme activity. These findings suggest that the development of pulmonary hypertension in infants with congenital cyanotic disease is not due to decreased expression of endothelial nitric oxide synthase, but instead may be related to a decreased ability of the enzyme to produce sufficient nitric oxide. (J Thorac Cardiovasc Surg 1998;115:343-50)**

Pulmonary hypertension is a common and serious manifestation of congenital cyanotic heart disease, yet its underlying pathophysiologic mechanism has not been fully elucidated. Historically, investigators have focused on the roles of increased pulmonary arterial flow or obstructed pulmonary venous flow and the development of arterial smooth muscle hypertrophy characterizing the hypertensive state. The molecular mechanisms underlying these physiologic events, however, were largely unpursued until the identification of nitric oxide (NO) as the endothelium-derived vascular smooth muscle relaxant described by Furchgott and Zawadski[1] in 1980.[2, 3]

NO is produced within endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS).[4] The reaction catalyzed by eNOS is dependent on the intracellular presence of calcium and calmodulin and also requires the presence of certain cofactors including tetrahydrobiopterin and reduced nicotinamide adenine dinucleotide phosphate (NADPH).[5]

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The NO thus produced diffuses to nearby vascular smooth muscle cells, where it causes a G-protein–mediated increase in cyclic guanosine monophosphate, which in turn leads to smooth muscle cell relaxation and thus vasodilatation.[6] Because of its role as a potent vasodilator and subsequent findings that inhibition of its production enhances the development of hypoxia-induced pulmonary vasoconstriction, a deficiency in the production of NO has been hypothesized to play a role in the development of pulmonary hypertension in the setting of chronic hypoxia.[7-9]

Whether this decrease in NO production is due to decreased expression of eNOS has been studied in both human beings and rats, but the results of these investigations have been generally inconclusive.[10-13] Furthermore, most of these studies have involved adult models. Our laboratory is specifically interested in the relationship between eNOS expression and pulmonary hypertension as it applies to infants with congenital cyanotic heart disease. Our aim in this study was to develop a large-animal infant model of hypoxia-induced pulmonary hypertension and to determine the levels of pulmonary eNOS protein and activity within this model. We hypothesized that hypoxia-induced pulmonary hypertension in infants is due to a decrease in the production of NO, either through a reduction in the expression of pulmonary eNOS or through a decrease in this enzyme's activity.

## Materials and methods

**Study groups.** Eleven 4-week old De Kelb piglets (Walnut Hill Farm, Hillsborough, N.C.) were studied. Six of these piglets were raised from 2 days of age at an inspired oxygen percentage of 10% (hypoxic group). The remaining piglets were raised in 21% oxygen (control group). Study group assignments were made in a randomized fashion. Piglets in the hypoxic group were placed at 2 days of age in a stainless-steel chamber and maintained in an atmosphere of 10% oxygen and 90% nitrogen, with air flow through the chamber being maintained at 16 L/min. The oxygen content within the chamber was monitored at all times (Beckman E.C, Oxygen Analyzer, Beckman Instruments, Inc., Fullerton, Calif.). Soda lime was placed in the chamber to prevent deleterious carbon dioxide accumulation. These animals were exposed to room air conditions for a mean of 20 minutes during each 24-hour period for replenishment of food supply and maintenance of the chamber. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 85-23, revised 1985) and were housed in the institution's National Institutes of Health–approved animal facility.

**Data acquisition.** At 4 weeks of age, anesthesia was induced in each animal with an intramuscular injection of ketamine (50 mg/kg) and acepromazine (15 μg/kg). Intravenous methylprednisolone (30 mg/kg) was administered via an 18-gauge cannula in the marginal vein of the pinna. Orotracheal intubation was performed and mechanical ventilation (Infant Ventilator; Sechrist Industries, Anaheim, Calif.) was commenced to achieve an arterial oxygen tension of 150 to 250 mm Hg and an arterial carbon dioxide tension of 35 to 45 mm Hg. The animals were paralyzed with intravenous pancuronium (300 μg/kg) and anesthetized with fentanyl (100 μg/kg). Thereafter, anesthesia was maintained with a continuous infusion of fentanyl (25 μg/kg per hour). An 18-gauge cannula was placed in the left femoral artery for blood pressure monitoring and arterial blood sampling. The heart was exposed through a median sternotomy. Cardiac instrumentation consisted of micromanometers (Millar Instruments, Inc., Houston, Tex.) inserted into the left atrium and the main pulmonary artery and a 10 mm ultrasonic flow probe (Transonic Systems, Ithaca, N.Y.) around the proximal pulmonary artery.

**eNOS protein analysis.** A peripheral pulmonary biopsy specimen was obtained from each animal and snap frozen in liquid nitrogen after storage at −80° C. A portion of the tissue from each hypoxic and control animal was later thawed, weighed, and homogenized in 150 μl ice cold tromethamine (Tris, 50 mmol/L) containing phenylmethyl-sulfonylfluoride (0.2 mg/ml), leupeptin (0.5 μg/ml), and 0.1% octyphenoxy polyethoxyethanol (Triton X-100, Union Carbide Corp., Canbury, Conn.). The homogenate was then lysed by sonication (Brinkman Instruments, Inc., Westbury, N.Y.), and pelleted by centrifugation (Brinkman Instruments). Total protein concentration of the supernatant was then determined by the Bradford assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.[14]

Equal samples of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 9% polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes for Western blot analysis. The membrane was then blocked in 7.5% nonfat milk in tromethamine-buffered saline solution/polysorbate (Tween) and then incubated with primary monoclonal anti-eNOS antibody at the manufacturer's specified concentration (Transduction Laboratories, Lexington, Ky.) for 1 hour at room temperature. eNOS protein bands were then visualized with antimouse immunoglobulin G horseradish peroxidase-conjugated antibody followed by chemiluminescence (DuPont, Boston, Mass.). The relative quantities of eNOS within the visualized protein bands were then quantified by laser densitometry (LKB Ultroscan XL, LKB Instruments, Inc., Rockville, Md.). Relative protein quantity was expressed as the percentage abundance in the control animals.

**NOS activity.** So that NOS activity in the lung tissue could be determined, samples from each biopsy were lysed by sonication in ice-cold, freshly prepared assay buffer containing tromethamine (Tris, 20 mmol/L), pH 7.4, ethylenediaminetetraacetic acid (EDTA, 1 mmol/L), and protease inhibitors soy trypsin inhibitor (100 μg/ml), N$\alpha$-*p*-tosyl-L-lysine chloro-methyl keatone (TLCK, 50 μg/

ml), phenylmethylsulfonyl fluoride (200 μg/ml), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), aprotinin (1 μg/ml), and antipain (50 μg/ml). So that NOS activity would be preserved, the buffer also contained NOS cofactors flavin adenine dinucleotide (5 μmol/L), flavin mononucleotide (5 μmol/L), biopterin (7 μmol/L), and diphtheria tetanus toxoid (DTT, 1 mmol/L). The cellular debris were then pelleted by centrifugation and 10 μl of the remaining lysate was added to 100 μl of a pre-prepared assay buffer mix containing calmodulin (150 nmol/L), calcium dichloride (1.75 mmol/L), biopterin (7.3 μmol/L)/DTT (1.33 mmol/L), $^3$[H]-arginine (1 μCi; $2 \times 10^6$ dpm [disintegrations per minute]), and NADPH (1 mmol/L), which was added last. The assay buffer and lysate sample were incubated at 30° C for 10 minutes, and the reaction was then stopped with the addition of 2 ml of stop solution (HEPES, 20 mmol/L; pH 5.5; EDTA, 2 mmol/L). The reaction wells were then washed with 2 ml of stop solution and the total mixture was then poured over a 1 ml silver 50W-X8 (sodium form) resin column (Bio-Rad 143-1451, Bio-Rad Laboratories), which had been pre-washed with 2 ml of stop solution. The flow through was collected and then the column was washed with an additional 2 ml of stop solution. Eluate (500 μl) was placed in scintillation vials quantified by liquid scintillation spectroscopy. The sample used for background calculation was processed in exactly the same manner, whereas the sample used for total counts was added directly to stop solution without column purification. The proportion of arginine that was convened to citrulline (and therefore the production of NO) was calculated as follows: (Disintegrations per minute in sample – Background disintegrations per minute)/(Total disintegrations per minute). To convert this proportion to moles of NO, we calculated the total quantity of arginine in the assay from the total disintegrations per minute (with a factor of 8 since only 500 μl was counted) and the known specific activity of $^3$[H]-arginine. NOS activity was then expressed as picomoles NO produced per milligrams of tissue per minute of incubation.

**Statistical analysis.** The pulmonary hemodynamic variables, eNOS protein levels, and NOS activity between the control and hypoxic groups were compared by nonpaired Student's *t* tests. All results are expressed as mean ± standard error of the mean. eNOS protein levels are expressed as a percentage of abundance in normoxic control lung.

## Results

**Study groups.** Eleven 4-week-old piglets were studied, with six being raised from 2 days of age at an inspired oxygen fraction of 10% (hypoxic group, $n = 6$) and the remainder being raised breathing room air (control group, $n = 5$). At the time of in vivo data acquisition, all pigs were mechanically ventilated at a normoxic inspired oxygen fraction. No discernible difference was observed between the two groups with regard to mean weight (6.52 ± 0.89 kg for hypoxic group vs 7.80 ± 0.19 kg for control group, $p = 0.232$), mean heart rate (117.9 ± 8.0 beats/min for hypoxic group vs 127.3 ± 6.7 beats/min for control group, $p = 0.392$), or mean arterial pressure (73.6 ± 11.6 mm Hg for hypoxic group vs 68.6 ± 2.0 mm Hg for control group, $p = 0.693$). The cardiac index was also similar for the two groups, averaging 80.2 ± 5.4 $ml \cdot min^{-1} \cdot kg^{-1}$ in the hypoxic piglets and 91.3 ± 14.3 $ml \cdot min^{-1} \cdot kg^{-1}$ in the control piglets ($p = 0.499$). Although the mean hematocrit value of the hypoxic animals was significantly greater that of the control animals (39% ± 2% in the hypoxic group vs 27% ± 1% in the control group, $p = 0.003$), no difference was detected in the oxygen saturations of the two groups while they were breathing room air (0.999 ± 0.006 in the hypoxic group vs 0.999 ± 0.002 in the control group, $p = 0.207$).

**Table I.** *Pulmonary hemodynamic variables and ventricular weights for the hypoxic and control groups*

| *Variable* | *Hypoxic (n = 6)* | *Control (n = 5)* | *p Value* |
|---|---|---|---|
| PVR (dyne·cm·sec$^{-5}$) | 7272.0 (871.1) | 1844.5 (271.2) | 0.002 |
| MPAP (mmHg) | 44.0 (3.8) | 14.8 (1.2) | 0.0007 |
| RV mass (gm) | 20.4 (2.7) | 7.3 (0.2) | 0.008 |
| LV mass (gm) | 20.1 (1.9) | 22.4 (1.3) | 0.344 |
| RV/LV mass ratio | 1.00 (0.05) | 0.33 (0.01) | 0.0002 |

Pulmonary hemodynamic data were measured in vivo during normoxic mechanical ventilation. The increases in mean pulmonary arterial pressure and pulmonary vascular resistance in the hypoxic animals indicate the development of substantial pulmonary arterial hypertension. Postmortem examinaiton of ventricular mass indicates a hypertrophic right ventricular response to the increased pulmonary pressures in the hypoxic piglets. All values are expressed as mean ± standard error of the mean. *PVR,* Pulmonary vascular resistance; *MPAP,* mean pulmonary arterial pressure; *RV,* right ventricular; *LV,* left ventricular.

**Pulmonary hemodynamics and ventricular weights.** While undergoing mechanical ventilation at normoxic conditions, the hypoxic animals had significant increases in both mean pulmonary vascular pressure and pulmonary vascular resistance when compared with the control animals (Table I). The right ventricular masses were significantly greater in the hypoxic animals than in the control animals, but the left ventricular masses did not differ. This resulted in a threefold increase in the right ventricle/left ventricle weight ratio in the hypoxic animals (Table I).

**Pulmonary eNOS analysis.** When the 153 kd eNOS bands obtained from Western blot analysis (Fig. 1) were quantified by laser densitometry, we found that the pulmonary eNOS levels were increased 2.01-fold in the hypoxic animals when compared with the control animals (Fig. 2). Despite the

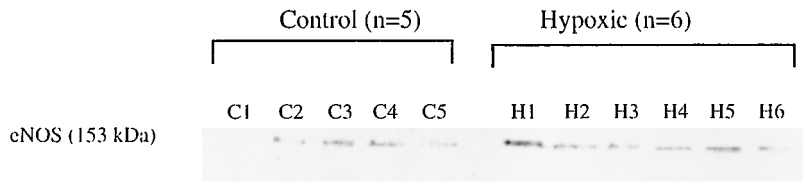


**Fig. 1.** Laser scan of Western blot showing the 153 kd eNOS bands in the control and hypoxic animals.

increased levels of eNOS in the lungs of the hypoxic animals, the total NOS activity per milligram of lung protein did not differ between the two groups (Fig. 3).

## Discussion

In this study, we show that raising newborn piglets in an environment of 10% oxygen for 4 weeks results in pulmonary arterial hypertension as evidenced by significant increases in main pulmonary arterial pressure and pulmonary vascular resistance in comparison with age-matched piglets raised in room air. A consequence of this pulmonary arterial hypertension was the development of right ventricular hypertrophy, a finding that further validates our model of chronic neonatal hypoxia. These changes in the pulmonary hemodynamics of the hypoxic piglets took place without a significant alteration in cardiac index, suggesting that hypoxia alone can produce pulmonary hypertension without the presence of increased flow through the pulmonary vessels.

Western blot analysis on lung biopsy tissue from all study animals using specific anti-eNOS antibody revealed that the hypoxic piglets had twofold more pulmonary eNOS than the control piglets, suggesting that neonatal pulmonary eNOS expression is up-regulated in the setting of chronic hypoxia. We then performed in vitro assays on the lung samples for the conversion of $^{3}$[H]-arginine to $^{3}$[H]-citrulline to determine the activity of the NOS present within the lungs. The assay was performed in room air with an excess of L-arginine and all the cofactors used by the enzyme. We found no significant difference in the activity of NOS per milligram of lung protein between the hypoxic and control groups. Inasmuch as the enzyme concentration was 101% higher in the hypoxic animals, a corresponding increase in the activity of NOS per milligram of lung protein would be expected. The absence of such a proportional increase suggests that the intrinsic ability of the enzyme to produce NO is impaired in the hypoxic animals. One possible explanation for this decrease in NOS activity is that the hypoxic piglets had reduced levels of molecular oxygen, a crucial substrate in the production of NO by the synthase enzyme.[15] Similarly, a reduction in the availability of L-arginine, the other substrate in the NOS reaction, may also account for the decreased production of NO.[16] However, such deficiencies do not explain the in vitro reduction in NOS activity that we demonstrate because this assay was performed in room air with an excess of L-arginine. This indicates that the eNOS enzyme itself may be functionally impaired in chronic hypoxia.

Our finding of increased pulmonary expression of eNOS production in the chronically hypoxic infant swine contrasts with results from anoxic cultured human vascular endothelial cells[17] and in adult patients with pulmonary hypertension of various causes,[13] both of which have shown a reduction in the expression of eNOS messenger ribonucleic acid. These results would suggest that chronic hypoxia compromises the ability of pulmonary vascular endothelium to produce NOS. However, studies in adult rats support our findings of overexpression of pulmonary eNOS in chronic hypoxia. For example, exposing adult rats to hypoxia for extended period of time has repeatedly been shown to result in increased levels of both eNOS messenger ribonucleic acid and protein.[10-12] Other investigations have shown that the pulmonary hypertension in adult rats can be significantly reduced if the rats are exposed to low levels of inhaled NO during hypoxia, indicating that the production of NO is deficient despite an overexpression of the enzyme that produces it.[18] Although neonatal piglets are clearly different from human infants, swine were chosen in this model because of their anatomic and physiologic similarities to human beings.

Although our model shares many of the advantages of the adult rodent model, especially the ability to expose the experimental subjects to low oxygen levels in a standardized laboratory setting, an important difference is that our model attempts to

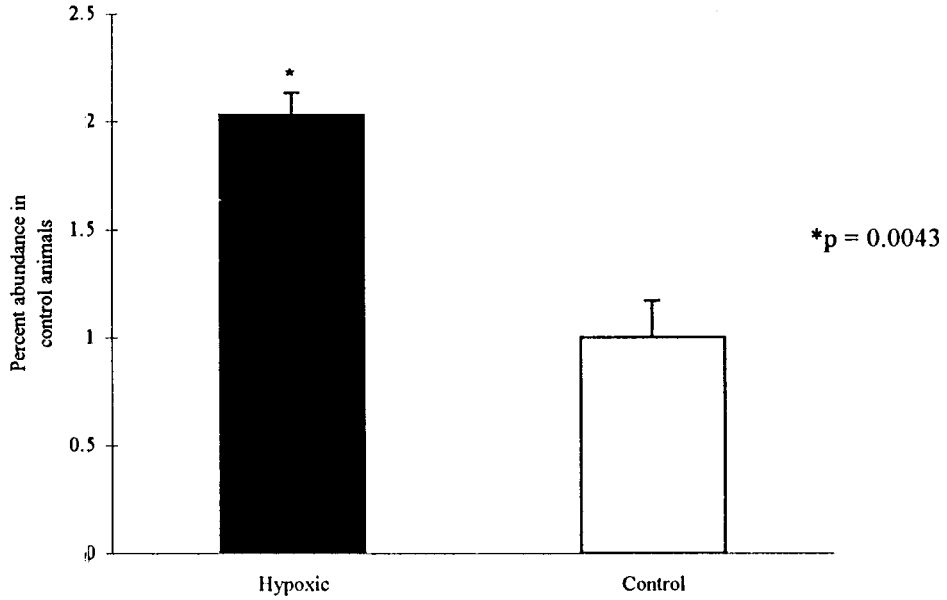


**Fig. 2.** Quantitative densitometry of the protein bands obtained from Western blot analysis revealed a twofold increase in eNOS levels in the lungs of the hypoxic piglets. Protein levels are expressed as percentage of abundance in the control animals. Statistical analysis was performed by means of the Student's *t* test.

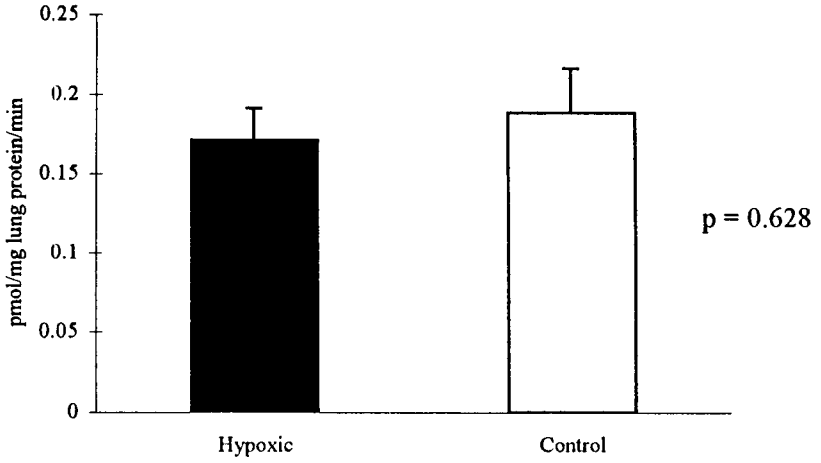


**Fig. 3.** NOS activity was determined by means of the $^{3}$[H]-arginine conversion assay, with the results being expressed as rate of activity per milligram of total lung protein. There was no detectable difference in the total NOS activity between the two groups.

explain the relationship between hypoxia-induced pulmonary hypertension and eNOS expression in an infant model. Because the endothelium-dependent vasodilatory capabilities of pulmonary vessels are likely to show maturational variations,[19, 20] it is important to use an infant model when examining the relationship between eNOS and pulmonary hypertension caused by congenital cyanotic heart disease. Recent investigations of isolated lungs from chronically hypoxic neonatal swine have shown a dampened response of these lungs to the endothelium-dependent vasodilatory agent acetylcholine when compared with age-matched normoxic control animals.[21] When exposed to *N*-nitro-L-arginine methyl ester (L-NAME), a known inhibitor of NOS,[22] the lungs from the control animals showed

significant vasoconstriction whereas the hypoxic lungs showed no response. These results suggest that the production of NO in the lungs of the hypoxic swine is reduced, thus supporting our finding of decreased eNOS activity in chronically hypoxic newborn piglets.

Importantly, our study addresses the effects of chronic hypoxia on the expression of pulmonary eNOS in newborn infants. We demonstrate that the development of hypoxia-induced pulmonary hypertension in neonates is associated with an overexpression of pulmonary eNOS, indicating that the ability of the pulmonary vascular endothelium to support the production of this enzyme is not limited by chronic hypoxia, and may in fact be stimulated by chronic hypoxia. Furthermore, we have shown that the ability of this enzyme to produce NO may be reduced in the setting of chronic hypoxia and that this decrease in enzyme function may contribute to the development of pulmonary hypertension. Therefore, efforts in treating infants with pulmonary hypertension associated with cyanotic heart disease should be directed toward understanding the mechanisms of decreased eNOS activity and increasing the production of NO.

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## Discussion

**Dr. Frank L. Hanley** *(San Francisco, Calif.).* It appears that you have created a nice neonatal large-animal model of chronic hypoxic pulmonary hypertension. It also seems clear that in this model, eNOS is elevated in the hypoxic animals. Beyond that is a great deal of room for speculation and discussion. This is a very interesting area for research with much potential clinical application. I encourage you and your group to continue to work in this area.

I have three separate areas for further discussion. The first relates to interpretation of your data. You have shown that eNOS doubles, but interestingly, when you

take the enzyme and challenge it in vitro to determine how much NO it actually makes, it does not appear to make any more than the samples from the control population. Have you evaluated this any further? Is the actual enzyme itself altered or is an associated inhibitor in your lysate competing with the enzyme. Along those lines, are you implying any kind of cause and effect with the pulmonary hypertension? Pulmonary vascular tone has multiple regulators. Is it possible that your elevated eNOS is simply a marker of other mechanisms and this elevation is simply a reaction to increased vasoactive tone caused by multiple other factors?

**Mr. Scarborough.** Those are very good points. The activity assay that we used has a couple of limitations that should be noted. First, it is an in vitro assay, which takes the process of NO production away from the body and the in vivo circumstances. Second, the assay is not specific for the endothelial isoform, so we cannot say that eNOS per se is not significantly increased. However, we do not think a reduction in another isoform's activity is masking an increase in the enzyme's activity. We are currently doing some follow-up studies to investigate why the activity of NOS may be decreased. A couple of possibilities that have been suggested in prior literature are possible deficiencies in the substrates L-arginine and molecular oxygen. Because our in vitro assay was performed with an excess of these substrates, we believe that deficiencies in these substrates are not a likely cause. Other explanations include the cofactors; one of particular interest is tetrahydrobiopterin. Although we have not examined this relationship further, some of the literature on coronary atherosclerosis has speculated that a deficiency in tetrahydrobiopterin may reduce the enzyme's activity and that these reductions may not be correctable merely by adding the cofactor in its preformed form into an in vitro assay. We would like to investigate that further. Also, we have undertaken a similar study in 2-week-old hypoxic piglets in which the vasodilatory response to acetylcholine in 2-week-old hypoxic animals was much less than in 2-week-old control animals, an observation that does seem to back up our findings in the in vitro assay that we used for the 4-week-old animals. It is, however, possible that there is an enzyme in the lysate that somehow interferes with the enzyme's ability to produce NO.

**Dr. Hanley.** The second point for discussion relates to methods. You have shown that the enzyme is doubled in the hypoxic tissue from your biopsy. Have you considered the following potential problem? These are neonatal piglets that have hyperplastic potential in their lung tissue and lung vascular tissue. Have you done any morphometric studies or assays of other endothelial protein to rule out the possibility that you are stimulating more vasculature here, and therefore, when you get 1 gm of lung tissue, you may actually have twice as much vessel and twice as much endothelium so when you look at the samples, you find you have twice as much eNOS but not twice as much expression?

The third point relates more to the appropriateness of the model. You emphasized that you were trying to mimic cyanotic congenital heart disease with this model. I would argue that you have created a very nice model for mountain sickness, but breathing thin air is not a model of cyanotic congenital heart disease. These animals are breathing lower oxygen concentrations, but they have no intracardiac mixing and they have absolutely normal pulmonary blood flow. What their vascular endothelium is seeing in your model is very different from that in the example of cyanotic congenital heart disease clinically. Cyanotic animals are hypoxemic because of mixing and sometimes reduced pulmonary blood flow, but the blood that does go through the microvasculature of the lung is no different than in you or me. A normal individual breathes in room air and oxygenates it; a cyanotic individual does the same thing. Your model is very different. This is still a very important study, but to say that it is an example of what happens in cyanotic congenital heart may well not be correct. I would like your input on this observation.

**Mr. Scarborough.** The alternative to our method of producing pulmonary hypertension would be to try to construct a shunt in a newborn piglet; we avoided that because we thought it might introduce too many other variables of injury that would cloud the issue. In developing our model, we used as our primary basis the work of another Duke group headed by Dr. James Lowe, who had constructed this hypoxic model to look at myocardial contractility in chronic hypoxia. Our method of producing chronic hypoxia is consistent with those of other investigators studying the effects of hypoxia on eNOS production.

**Dr. Hanley.** I think you are doing an excellent job in a complex area that has great potential for clinical application, and I would encourage you to continue this work.

**Dr. David A. Fullerton** *(Winnetka, Ill.).* I would like to echo some of the comments made earlier. The model you have used is a standard model to develop pulmonary hypertension. Work published in the early 1990s nicely demonstrated in sheep that throughout the newborn period for approximately 2 weeks after birth the amount of eNOS is diminished. It is part of the maturation problem, and it is thought that this may contribute to higher pulmonary arterial pressures in newborn infants. Work has shown that through augmented expression of vascular endothelial growth factor, there is a proliferation of capillaries including endothelial cells. Do you have any histologic studies looking at neovascularization as part of your explanation for some of your findings?

**Mr. Scarborough.** We do have specimens for histologic studies. However, we have not looked at those and we have not done morphometric analysis at this time.

**Dr. Fullerton.** It is paradoxical that you find elevated levels of eNOS in the presence of very high pulmonary arterial pressure. I realize that is the whole crux of your issue. It turns out that there is a strain of rats that spontaneously become pulmonary hypertensive, and they too share this paradox; they have high levels of eNOS, yet very high pulmonary arterial pressures. Could you speculate on how this happens? Is it possible that the eNOS is a compensatory response to the hypoxia-induced pulmonary hypertension?

**Mr. Scarborough.** That would make sense, given the role of eNOS in producing NO, which then vasodilates the vessels. There could be an adaptational up-regulation. There may also be some circumstances surrounding chronic hypoxia that would prevent the enzyme from

doing its job, although the same could be said theoretically about a decrease in eNOS enzyme itself causing pulmonary hypertension. That, I believe, has been the contrast in the past, trying to decide which one of those is the case. I think enough studies have been conducted to suggest that eNOS expression probably is increased. Studies in brown humpback rats also lend support to that. The goal now is to evaluate at the molecular level the reason that the enzyme is not producing NO.

**Dr. Hanley.** Just one last comment: We are trying to get around this cause-and-effect thing. If your house is not on fire, there usually is not much water on it; if your house *is* on fire, there is an increase in the amount of water on your house, but that is not a paradox. In the same way, if a lung is chronically hypoxic and vasoconstricted, the increase in eNOS is just a reaction, even though eNOS is a vasodilator. I do not find this paradox very difficult to understand myself.

**Mr. Scarborough.** That is a good point. In fact, we believe that we have shown that the pulmonary vascular endothelium has retained its ability to produce NO despite the hypoxic environment.

**Dr. Fullerton.** Does endothelin have an effect? Have you measured any of that in the model?

**Mr. Scarborough.** We have not measured endothelin in this model. That would be a good idea for future studies.